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13. ABSTRACT *(Maximum 200*

The proposal of my grant is to investigate the biological significance and mechanism of insulin-like growth factor binding protein-3 (IGFBP-3) as well as identification and characterization of the IGFBP-3 receptor in human breast cancer cells. As a second year task, I have successfully screened the IGFBP-3 interacting proteins including putative IGFBP-3 receptor in human breast cancer cells by employing the yeast two-hybrid system. Two cDNA clones matched sequences in the GenBank database: (1) Eps8 - epidermal growth factor receptor kinase substrate, and (2) GRP78 / BiP - glucose regulated stress protein, or human immunoglobulin heavy chain binding protein. The third cDNA, designated clone 4-33, was not identified in the database and represents a novel gene / protein. I have thus far concentrated my efforts on this novel cDNA clone: (1) synthesis of recombinant human 4-33 protein by use of a Glutathione S-Transferase fusion and (2) generation of polyclonal 4-33 antibodies. Proceeding with our research for IGF-independent action of IGFBP-3 in breast cancer cells, I have identified and characterized connective growth factor as a member of low-affinity IGF binders, IGFBP-related protein 2 (IGFBP-rP2). Further investigation revealed that IGFBP-rP2 is expressed in normal mammary cells as well as breast cancer cells but with significantly reduced level in estrogen-responsive breast cancer cells. Its expression is regulated by transforming growth factor-beta (TGF-β) as similarly observed in IGFBP-3. These studies indicate that those IGFBP-rPs show similar characteristics in their biological actions, which are comparable to those of IGFBP-3 in terms of breast cancer cell growth inhibition. Therefore, in combination with the IGFBP-3/IGFBP-3 receptor studies I am also investigating these new potent growth inhibitors of breast cancer cells for inclusion as members of IGFBP superfamily.

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FOREWORD

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Yoonjoo Oh 10-23-98

PI - Signature Date

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I. INTRODUCTION.

The insulin-like growth factor binding proteins (IGFBPs) 1-6 bind IGF-I and IGF-II with high affinity and serve to transport the IGFs, prolong their half-lives, and modulate their proliferative and anabolic effects on target cells (1-9). The molecular mechanisms involved in the interaction of the IGFBPs with the IGFs and their receptors remain unclear, but these molecules appear, at least, to regulate the availability of free IGFs for interaction with IGF receptors (10, 11). Recent studies from our laboratory and others demonstrated that some IGFBPs have ability to exert IGF-independent actions.

In this project, I proposed investigation of the biological significance and mechanism of this IGF-independent action of IGFBP-3 as well as identification and characterization of an IGFBP-3 receptor in human breast cancer cells. As a first year task, I initiated the identification of the putative IGFBP-3 receptor in human breast cancer cells by employing a yeast two-hybrid system. In addition, I have generated IGFBP-3 fragments using a baculovirus expression system for characterization of structure-function aspects of IGFBP-3 actions. In addition, we have identified mac25 as a new member of low-affinity IGF binder and named as IGFBP-related protein 1 (IGFBP-rP1). Further investigation has been executed to identify and characterize IGFBP-3 interacting proteins and other putative low-affinity IGF binders.

II. BODY.

1. Yeast Two-Hybrid Screen for the IGFBP-3 Receptor in Hs578T Human Breast Cancer Cells

I had initially proposed to employ expression cloning by use of lambda expression libraries for identification of an IGFBP-3 receptor. The expression library, consisting of Hs578T-derived cDNAs cloned into the lgt11 expression vector, was generated and screened using [^{125}I]IGFBP-3 or polyclonal IGFBP-3 receptor antibodies. However, I have faced technical difficulties due to the nature of the IGFBP-3 protein, specifically its ability to bind proteins in a non-specific manner under these experimental conditions. As an alternative approach, yeast two-hybrid screening was employed to identify IGFBP-3 interacting proteins (12).

As reported in the first year accomplishments, using intermediate fragment of IGFBP-3 (amino acids 88-148) as the "bait", we are screening a cDNA library made from an Hs578T mRNA preparation to pull out the putative IGFBP-3 receptor. Three individual cDNA clones demonstrated specificity for IGFBP-3 interaction when tested against lamin, a commonly used false-positive indicator (Figure 1). Two cDNA clones matched sequences in the GenBank database: (1) Eps8 - epidermal growth factor receptor kinase substrate, and (2) GRP78 / BiP - glucose regulated stress protein, or human immunoglobulin heavy chain binding protein. The third cDNA, designated clone 4-33, was not identified in the database and represents a novel gene / protein. I have thus far concentrated my efforts on this novel cDNA clone.

When the 4-33 cDNA clone was transiently expressed as a green fluorescent protein (GFP) fusion in COS-7 cells, the subcellular distribution was perinuclear or diffusely cytoplasmic, as compared to the nuclear and cytoplasmic expression of unfused GFP. The GFP::4-33 fusion protein was undetectable in the conditioned media of these transfected cells. Coimmunoprecipitation assays from cell lysates of COS-7 cells co-transfected with GFP::4-33 and IGFBP-3 cDNAs demonstrated a strong and specific interaction between the 4-33 protein and IGFBP-3 (Figure 2 and 3). Northern blot analysis revealed that the 1.0 kb mRNA for clone 4-33 is widely expressed in breast cancer cells and various human tissues, presenting the possibility that this protein may play a ubiquitous role in the IGF-independent actions of IGFBP-3 (Figure 4).

Further studies will uncover the significance of this novel protein in the biological actions of IGFBP-3. In the absence of an antibody to 4-33, we plan to express hemagglutinin- (HA-) tagged 4-33 in COS7 and human breast cancer cells for further investigation into subcellular localization and interaction with IGFBP-3. The HA system adds only 9 amino acids to the C-terminus of the 4-33 protein sequence, rather than the 240 amino acids of the GFP protein. Concurrently, we will also express and purify the 4-33 protein as a Glutathione S-Transferase (GST) fusion. This system allows large scale purification of the fusion protein with subsequent cleavage of GST, resulting in pure 4-33 protein. The protein will be useful for cellular, biochemical, and interaction studies with purified IGFBP-3 protein. In addition, we plan to use the purified 4-33 protein to generate a polyclonal 4-33 antibody which will be useful for investigating endogenous 4-33 protein in several cell systems, as well as additional biochemical and interaction studies.

2. Characterization of IGFBP-rP2, a New Low-Affinity Member of IGFBP Family

As reported last year, my laboratory has identified mac25 as IGFBP-rP1 using baculovirus expressed recombinant human mac25 and polyclonal antibodies specific for human mac25. Further efforts to identified other low-affinity IGF binders revealed that Connective tissue growth factor (CTGF) is another member of IGFBP superfamily.

CTGF is a cysteine-rich mitogenic peptide that was originally identified as a growth factor secreted by vascular endothelial cells in culture. The gene, residing on chromosome 6q23.1, proximal to c-*myb*, was cloned from human umbilical vein endothelial cells (13). CTGF family members, including the nov oncogene and Cyr61, are thought to regulate cell proliferation, differentiation, embryogenesis and wound healing (14). Although CTGF exhibits platelet-derived growth factor (PDGF)-like biological activities and appears to be antigenically related to PDGF, it has little peptide sequence similarity to either the PDGF A or B chain peptides (1). CTGF has 30-38% amino acid sequence similarity to IGFBPs and contains critical conserved sequences, such as the N-terminal GCGCCXXC motif and the N-terminal cluster of cysteines conserved in IGFBPs. Indeed, baculovirus generated CTGF, a secreted 38-kDa protein, binds IGFs specifically, leading

to its inclusion in the IGFBP superfamily and its provisional renaming as IGFBP-rP2 (Appendix #1). Several studies have shown that TGF-β is a potent stimulator of CTGF expression in human skin fibroblasts and chondrosarcoma-derived chondrocytic cells. Furthermore, analysis of deletion mutants indicated that a novel TGF-β responsive element is located between positions -162 and -128 of the CTGF promotor. Since TGF-β has been shown to inhibit proliferation of human breast cancer cells, as well as up-regulate expression of IGFBP-3, which appears to mediate the growth inhibitory action of TGF-β, we investigated the regulation of IGFBP-rP2 by TGF-β in Hs578T human breast cancer cells using recombinant human IGFBP-rP2^{bac} and polyclonal anti-IGFBP-rP2 antibody. In the present study, we demonstrate that expression of IGFBP-rP2 is upregulated by TGF-β in Hs578T human breast cancer cells. We further report that differentially glycosylated forms of IGFBP-rP2, ranging from 32- to 38-kDa, as well as degraded forms of 18- and 24-kDa, are detectable in various human biological fluids, suggesting that IGFBP-rP2 is an important endocrine factor *in vivo* and potentially involved in the growth inhibitory action of TGF-β in human breast cancer cells (Appendix #2). These data are particularly important to understanding IGF-independent actions of IGFBPs and the new concept of IGFBP superfamily.

III. CONCLUSIONS.

We have successfully executed our second year tasks (DAMD17-96-1-6204) outlined in the Statement of Work except Tsak 1. As mentioned in previous statements, we had difficulites to employ expression cloning techniques for identification of an IGFBP-3 receptor due to the nature of the IGFBP-3 protein, specifically its ability to bind proteins in a non-specific manner under these experimental conditions. As an alternative approach, yeast two-hybrid screening was employed to identify IGFBP-3 interacting proteins. Nevertheless, we have successfully screened the IGFBP-3 interacting proteins including putative IGFBP-3 receptor in human breast cancer cells by employing the yeast two-hybrid system. Two cDNA clones matched sequences in the GenBank database: (1) Eps8 - epidermal growth factor receptor kinase substrate, and (2) GRP78

/ BiP - glucose regulated stress protein, or human immunoglobulin heavy chain binding protein. The third cDNA, designated clone 4-33, was not identified in the database and represents a novel gene / protein.

In addition, we have successfully proceeded our first year tasks (DAMD17-97-1-7204) outlined in the Statement of Work: tasks in progress follow: (1) detection of the putative IGFBP-3 receptor, 4-33, mRNA expression by Northern blotting in normal and breast cancer cells (Task 1); (2) generation of an inducible stable transfection of IGFBP-3 and 4-33 in breast cancer cells (Task 2); and (3) synthesis of recombinant 4-33 protein and generation of polyclonal antibodies as mentioned above (Task 5 and 6).

Further investigation on the IGFBP superfamily resulted in identification of connective growth factor as a member of low-affinity IGF binders, IGFBP-rP2. IGFBP-rP2 is expressed in normal mammary cells as well as breast cancer cells but with significantly reduced level in estrogen-responsive breast cancer cells. Its expression is regulated by transforming growth factor-beta (TGF-β) as similarly observed in IGFBP-3. These studies indicate that those IGFBP-rPs show similar characteristics in their biological actions, which are comparable to those of IGFBP-3 in terms of breast cancer cell growth inhibition. Therefore, in combination with the IGFBP-3/IGFBP-3 receptor studies I am also investigating these new potent growth inhibitors of breast cancer cells for inclusion as members of IGFBP superfamily.

These studies will provide insights into the multiple actions of IGFBP-3, both IGF-dependent and IGF-independent. Depending on the presence of IGF peptide or the functional status of receptors, IGFBP-3 can exert biological actions either through its own receptor (IGF-independent) or by modulating IGF binding to IGF receptors (IGF-dependent), either of which may provide a mechanism for IGFBP-3 as an anti-proliferation factor in the human mammary system.

IV. REFERENCES

1. Salmon WD, Daughaday WH: A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J Laboratory Clinical Medicine 49:825-836, 1957.
2. Daughaday WH, Hall K, Raben MS, Salmon WD, Van den Brande JLV, and Van Wyk JJ: Somatomedin: Proposed designation for sulphation factor. Nature 235:107, 1972.
3. Daughaday WH, Rotwein P: Insulin-like growth factors I and II peptide messenger ribonucleic acid and gene structures serum and tissue concentrations. Endocrine Rev 10:68-91, 1989.
4. Rosenfeld RG, Lamson GL, Pham H, Oh Y, Conover C, DeLeon DD, Donovan SM, Ocrant I, Giudice LC: Insulin-like growth factor-binding proteins. Recent Progress in Hormone Res 46:99-159, 1991.
5. Baxter RC, Martin JL: Binding proteins for the insulin-like growth factors: structure, regulation and function. Progress Growth Factor Res 1:49-68, 1989.
6. Jones JL, Clemmons DR: Insulin-like growth factors and their binding proteins: biological actions. Endocrine Rev 16:3-34. 1995.
7. Oh Y, Muller HL, Neely EK, Lamson G, Rosenfeld RG: New concepts in insulin-like growth factor receptor physiology. Growth Regul 3:113-123, 1993.
8. Werner H, Woloschak M, Stannard B, Shen-Orr Z, Roberts CT Jr., LeRoith D: The insulin-like growth factor receptor: molecular biology heterogeneity and regulation. In: LeRoith (eds.) Insulin-like growth factors: Molecular and Cellular Aspects CRC Press Boca Rawton, 1991, pp. 17-47.
9. Lowe L: Biological actions of the insulin-like growth factors. In: LeRoith (eds.) Insulin-like growth factors: Molecular and Cellular Aspects CRC Press Boca Rawton ,1991, pp. 49-85.
10. Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson D, Rosenfeld RG: Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. Int J Biochem Cell Biol 28:619-637, 1995.
11. Jones JI, Clemmons DR: Insulin-like growth factors and their binding proteins: biological actions. Endocrine Review 16:3-34, 1995.

12. Fields S, Song O: A novel genetic system to detect protein-protein interactions. Nature 340:245-246, 1989.

13. Bradham DM, Igarashi A, Potter RL, Grotendorst GR: Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product. J Cell Biol 114:1285-1294, 1991.

14. Oemar BS, Luscher TF: Connective tissue growth factor Friend of Poe?. Arteriosclerosis, Thrombosis and Vascular Biology 17:1483-1489, 1997.

V. APPENDICES

1. Figure 1
2. Figure 2
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4. Figure 4

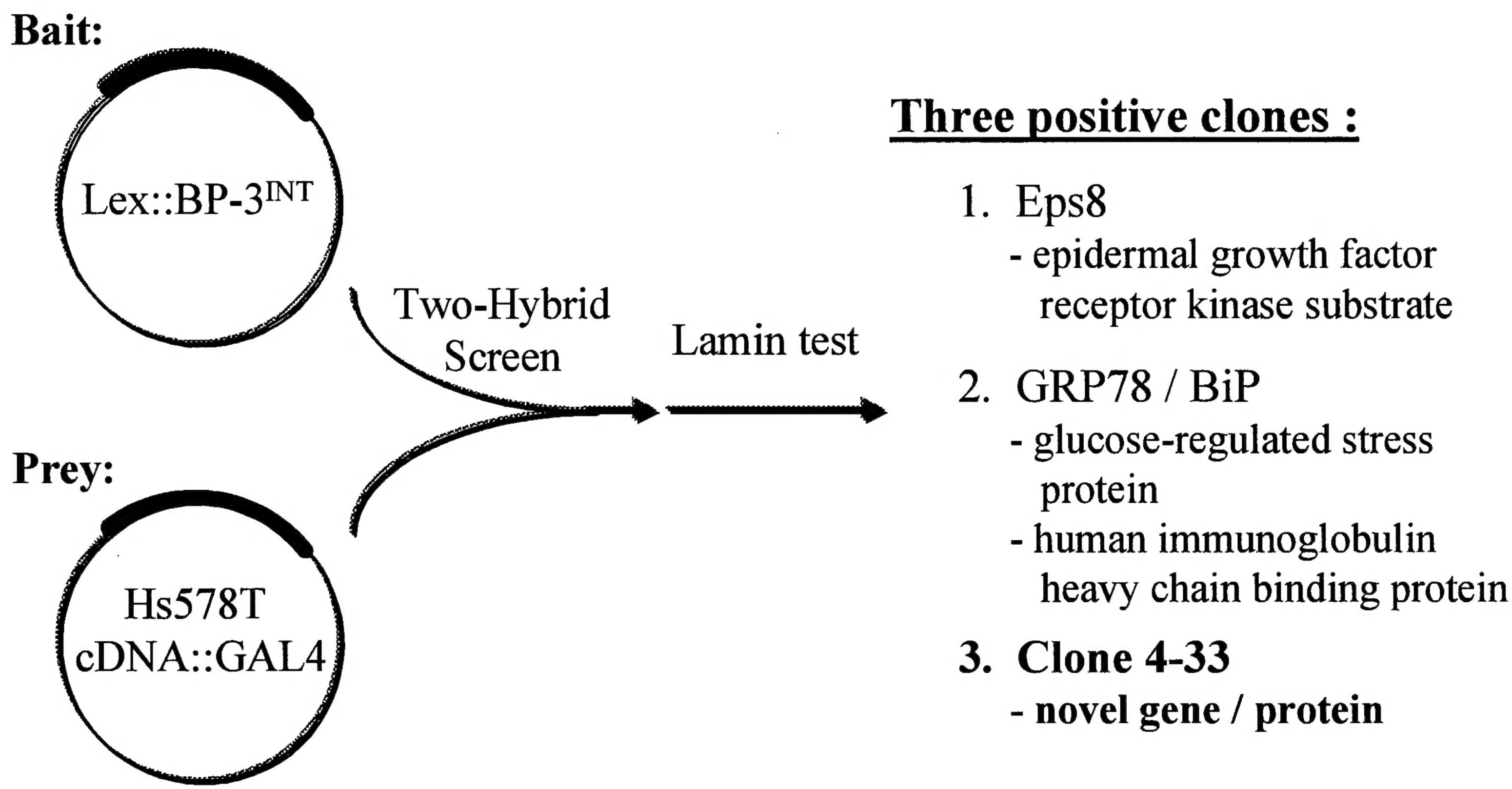

Figure 1. A portion of the IGFBP-3 cDNA, coding for an internal 61 amino acids, was fused to the Lex DNA binding domain. A cDNA library, generated from Hs578T human breast cancer cell mRNA, was fused to the GAL4 transcriptional activation domain. These were used in the yeast two-hybrid system to identify clones which potentially interact with IGFBP-3.

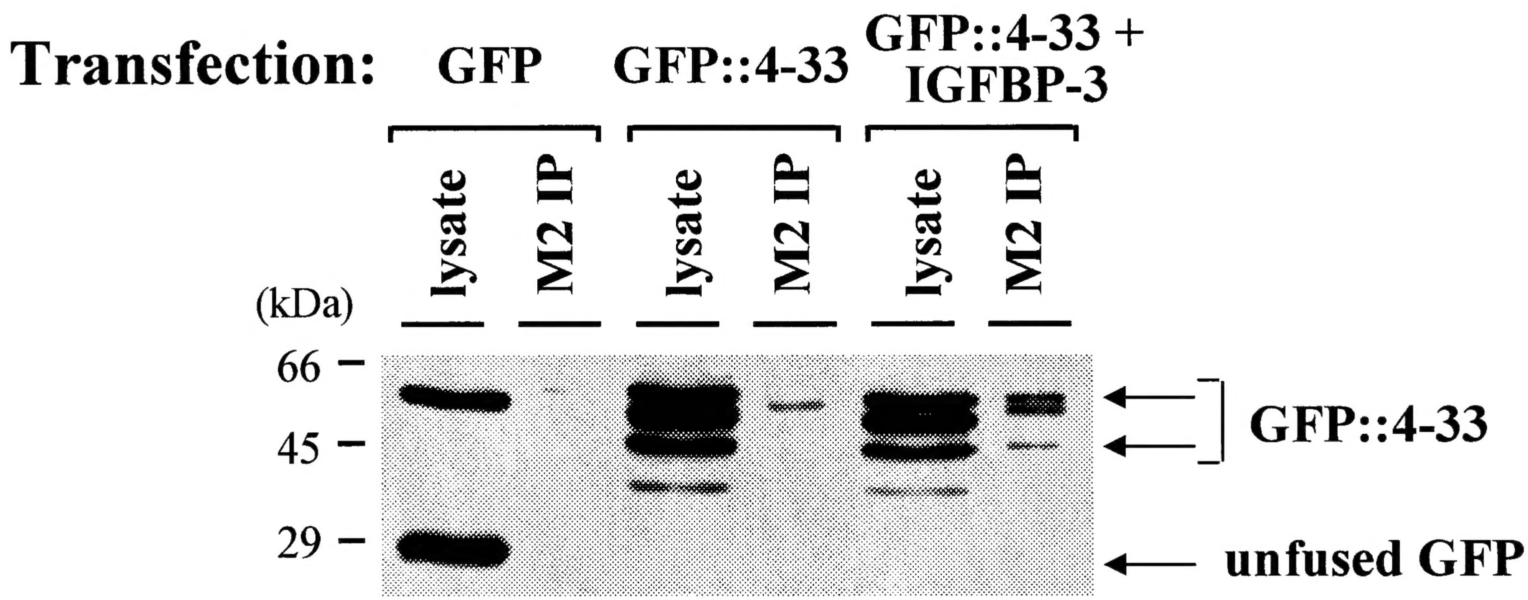

Figure 2. COS-7 cells were transiently transfected with expression constructs for GFP, GFP::4-33 and/or IGFBP-3 as indicated. After 36 hours, cell lysates were harvested and precleared by incubation with Anti-mouse IgG sepharose. Precleared lysates were then immunoprecipitated overnight with α Flag M2 affinity beads and subjected to SDS-PAGE under reducing conditions. Following transfer, WIB analysis was done with α GFP polyclonal Ab and ECL detection.

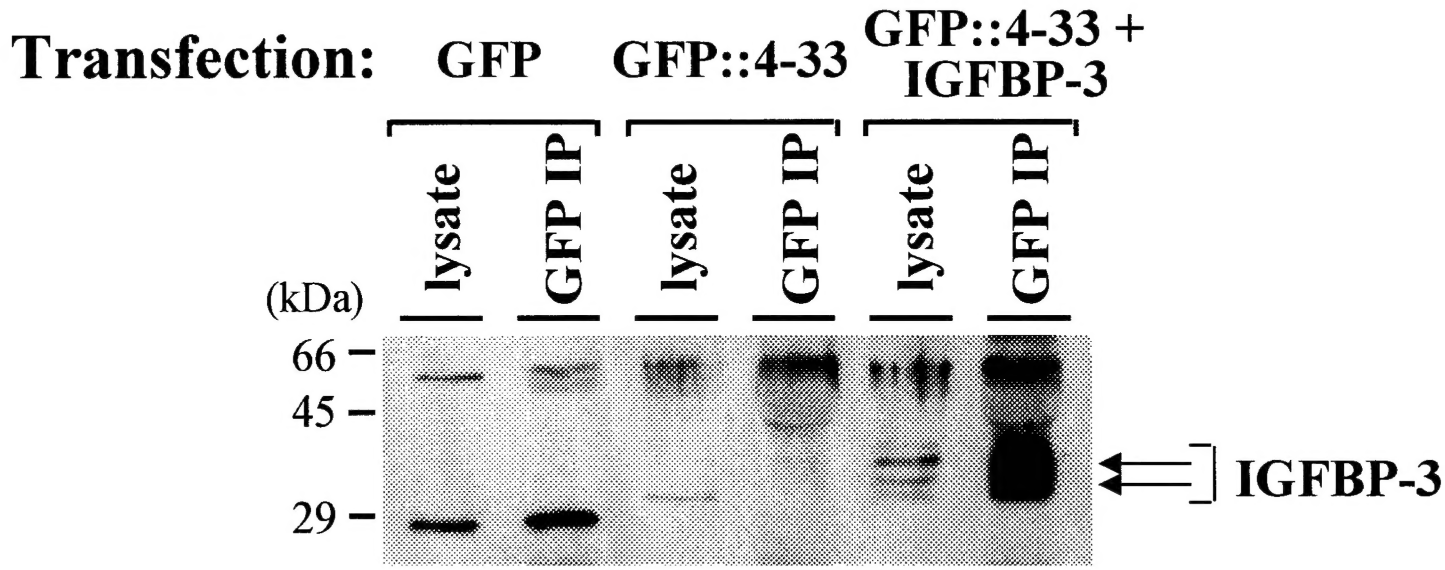

Figure 3. COS-7 cells were transiently transfected with expression constructs for GFP, GFP::4-33 and/or IGFBP-3 as indicated. After 36 hours, cell lysates were harvested and precleared by incubation with α-mouse IgG sepharose. Precleared lysates were then immunoprecipitated overnight with α GFP monoclonal Ab + α-mouse IgG sepharose and subjected to reducing SDS-PAGE. WIB analysis was first done with α GFP polyclonal Ab, and then with α IGFBP-3 polyclonal Ab.

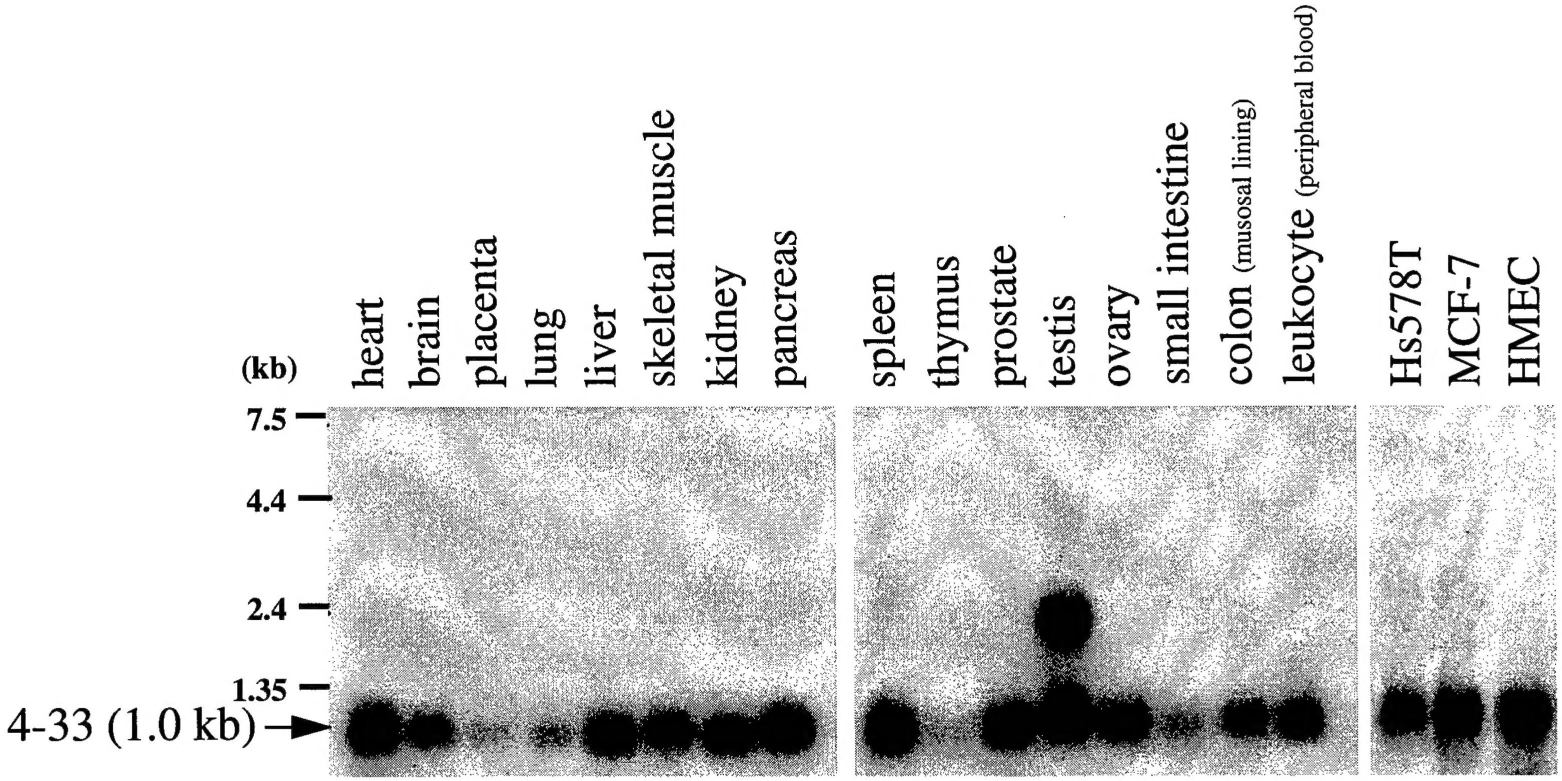

Figure 4 Northern analysis of the distribution of clone 4-33 in human tissues and cell lines. Human multiple tissue Northern blots I & II (Clontech), and Northern blots containing total RNAs from Hs578T and MCF-7 human breast cancer cell lines and human mammary epithelial cells (HMEC) were probed with the 1.0 kb cDNA from clone 4-33.